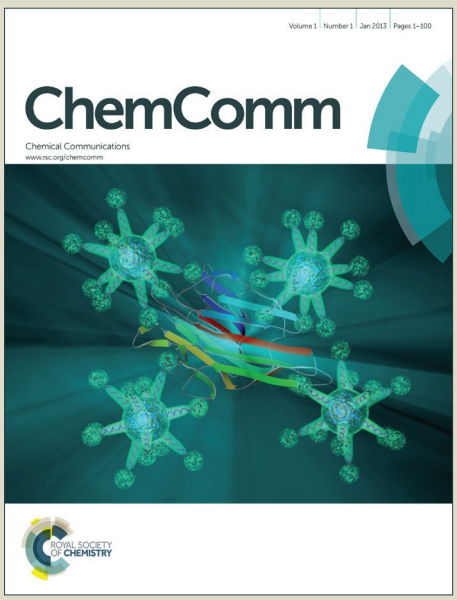
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Highly Chemoselective Ligation of Thiol- and Amino-Peptides on a Bromomaleimide Core

Suhas Ramesh,[a] Prabhakar Cherkupally,[a] Thavendran Govender,[a] Hendrik G. Kruger,[a] Fernando Albericio,[a,b,c,d*] Beatriz G. de la Torre[a*]

Received (in XXX, XXX) XthXXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Application of a bromomaleimide core allows for the incorporation of three different peptides. The key reactions of the process are the selective stapling of both thiol- and amino-peptides on two different sites of the core. The thiol-peptide attacks and replaces the bromide whereas the amino-peptide attaches to the ene-position of the core revealing differential and selective reactivity. This platform will have further application in protein chemistry, multidrug presentation and vaccine preparation.

The preparation of (bio)conjugates is a need in several scientific fields such as multidrug presentation,[1-3] peptide-based vaccine preparation[4,5], and protein chemistry.[6,7] Thus, the development of platforms able to incorporate several chemical structures has raised particular importance.[8-10] Tam in 1988 described the synthesis of multiple antigenic peptides (MAPs) as vaccine candidates, which was limited to multihomo-drug presentation.[11] In this context, recently we have described the synthesis and application of the 6-(bromomaleimido)hexanoic acid (BMHA) platform for the construction of multiple branched peptides.[12] In our work and that of Baker *et al.*[13-15], peptides are exclusively incorporated to the bromomaleimido core through the thiol of Cys. However, taking into account that the bromomaleimide ring has two reactive positions susceptible to nucleophilic attack, namely Br (nucleophilic substitution) and the *ene* (Michael addition) positions, we envisaged the possibility to introduce two different molecules with different functionalities to assure total regioselectivity. In addition to these, a third connection through the carboxylic acid of the BMHA can be explored to provide optimum diversity (Fig. 1).

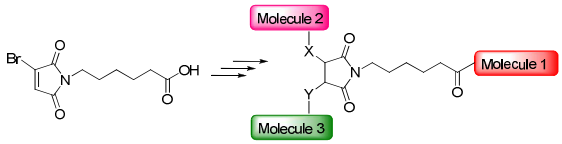


Fig. 1. Schematic representation of the (bio)conjugation of 3 different molecules on the BMHA core.

Taking into account that thiol and amino functions, which are present in a large number of biomolecules and commonly involved as nucleophiles, we attempted the selective incorporation of two peptides Ac-X-Leu-Ala-Gly-Val-NH_2 [X=Cys, (Peptide **2**), Lys (Peptide **3**)] onto BMHA-Tyr-Gly-Gly-Phe-Leu-NH_2 (Peptide **1**).[12]

The conjugation of thiol-peptide **2** with BMHA-peptide **1** (Fig. 2, Route A) was carried out at pH 6.5 in phosphate buffer by adding portion wise one equivalent of thiol-peptide **2** and monitoring the reaction by analytical HPLC. The reaction gave only one product and proceeded very fast and quantitatively. ^{1}H-NMR and MS of the obtained conjugate confirmed that thiol-Peptide **2** attacked at the bromo-substituted carbon and replaced Br through a nucleophilic substitution reaction (NMR, Fig 3, Panel B; MS, Fig. S3, SI).

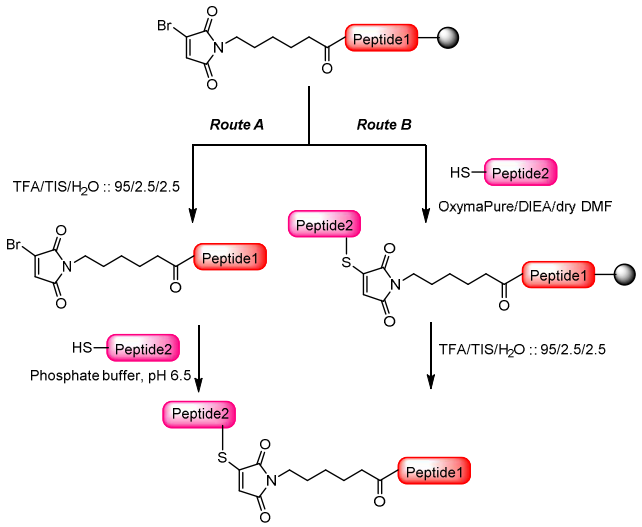


Fig. 2 Schematic representation of thiol-peptide conjugation by (A) solution-phase and (B) solid-phase approaches on bromomaleimide core

The same conjugation was carried out on solid-phase (Fig 2, Route B). In a first attempt, thiol-Peptide **2** in DMF/DIEA was added to BMHA-peptide **1**-ChemMatrix (CM) resin, after 1h the reaction was not completed although the main product was the conjugate. After extending the reaction time, no improvement was observed. Under these conditions oxidation of thiol-Peptide **2** competed with the nucleophilic substitution reaction[16,17]. Next,

we used the so-called organic buffers as a solvent to carry out the reaction. The use of OxymaPure[18,19] (pKa 4.60)/DIEA 1:1 in dry DMF yielded the required product in about 4h time, avoiding the formation of the thiol-Peptide 2 dimer. The conjugate obtained by both methods, solution and solid-phase, were purified by semi-preparative HPLC and characterized by LCMS (Fig S1-S3, SI).

To study the reactivity of amino-Peptide **3** with BMHA-Peptide **1** in solution (Fig 4, Route A), different basic media were screened. When 20 mM $NaHCO_3$ of pH 8 was assayed, instead of the desired conjugate a mixture of two hydroxylated products (3:1) was observed. Therefore, it was necessary to avoid aqueous medium and hence it was decided to use 0.1% DIEA in DMF/ACN, in which the reaction goes to completion rapidly. ^{1}H-NMR of the obtained conjugate confirmed that amino-Peptide **3** attacked at the ene-position of the core by means of a Michael addition reaction leaving bromide intact which is in contrast to thiol-conjugation (Fig 3, Panel C) (MS showed Br pattern, Fig S7, SI).

To perform this conjugation described before on solid-phase (Fig 4, Route B), the first trial was to reproduce the same conditions as in solution. Thus, 1 equivalent of amino-Peptide **3** was dissolved in 0.1% DIEA in DMF/ACN, added to BMHA-peptide **1** CM-resin and allowed to react for 1h. After cleavage and deprotection, again the major products obtained were those from hydroxylation reaction. Then the conjugation was repeated using only anhydrous DMF as solvent but any improvement was found. At this point we concluded that the only OH source is the ChemMatrix resin[20] used to prepare BMHA–peptide **1**-resin, thus the synthesis was repeated on a polystyrene (PS)-based resin. The reaction with this new BMHA-peptide **1** PS-resin using DIEA/dry DMF yielded the required conjugated compound (Fig. 4). The conjugate obtained by both methods was purified by semi-preparative HPLC and characterized by LCMS (Fig S4-S6, SI).

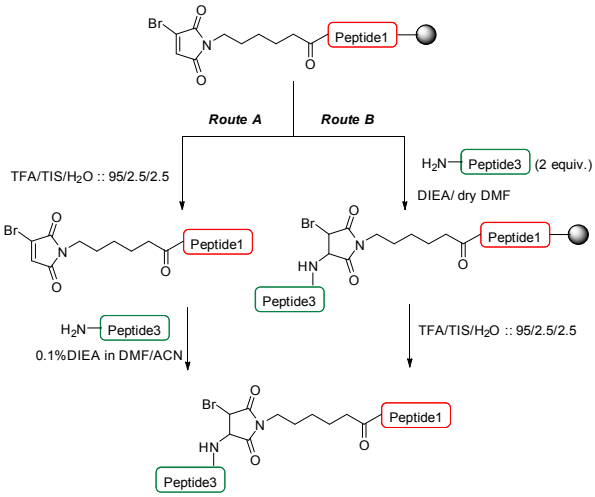


Fig. 4. Solution- and solid-phase approaches for amino-peptide conjugation on BMHA-peptide **1**.

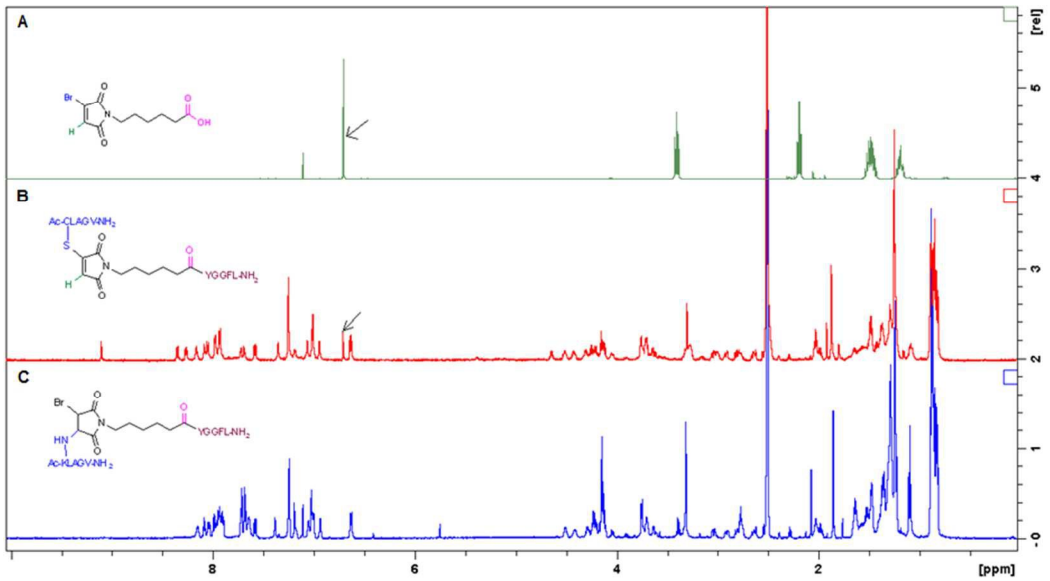


Fig. 3 Overlay of NMR spectra of bromomaleimide core (green), Cys-conjugate (red) and Lys-conjugate (blue) in which alkene signals appeared at δ 6.7 ppm (arrow marks) as observed in first two panels and absent in the last one.

After obtaining the first conjugates of both thiol- and amino-peptides separately, the next step was to attempt the double conjugation having thiol- and amino-functions on the same molecule. Three different examples of S- and N-nucleophiles on BMHA-peptide **1** were assayed: simple Fmoc-Cys/Lys amino acids, model peptides (thiol peptide **2** and amino peptide **3**), and finally a HIV-protease inhibitor (TLNF-OH and Ac-XTLNF-OH, X = C/K). Using the same conditions used for mono conjugation in solution, route A (Fig. 5), which involves first the S- and then the N-conjugation took place smoothly and rendered the target in all the three cases (Figs. 6 and S8-S9, SI). Next, route B (Fig. 5), which involves first the N- and then the S-conjugation was

performed. As expected, N-conjugation gave excellent yields in all three cases, but the conjugation of the thiol-component over the conjugate molecule **3**-NH-BMHA-peptide **1** was not observed, even after application of different conditions. In this case the Br position became less reactive since the substituted maleimide was previously converted into a substituted succinimide, which is less reactive toward thiol nucleophilic attack.

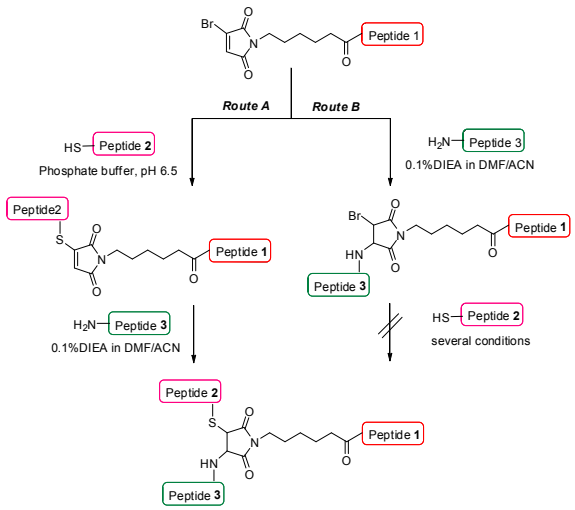


Fig. 5. Strategy for S-, N- bis-conjugation

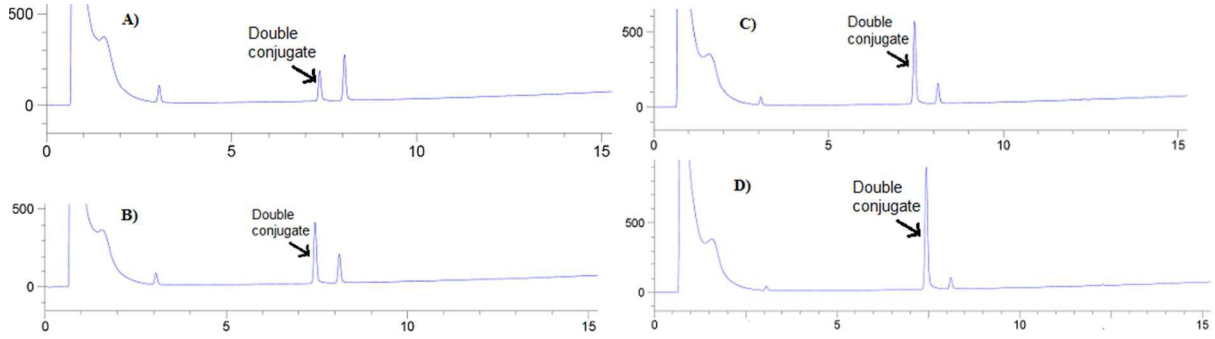


Fig. 6. HPLC traces of double conjugation using HIV protease inhibitor peptides at A) 1h B) 3h C) 7h and D) overnight.

The double conjugates obtained were characterized by HPLC and LCMS (Figs 6 and S8-S12, SI).

In conclusion, we have developed a rational, chemoselective, and unambiguous method for the preparation of a platform that is able to accommodate three different moieties. The core based on the 6-(bromomaleimido) hexanoic acid is able to incorporate (i) a molecule/peptide at the C-terminal through an amide bond carried out in solid-phase; (ii) a thiol-moiety at the bromide position, which can be carried out indistinctively in solution- or in solid-phase; and (iii) an amino-moiety at the *ene* position through a Michael addition carried out in solution. All reactions are performed under mild reaction conditions including temperature, pH and solvent compatibility, which prevent side reactions, such as hydroxylation and/or oxidation of the thiol-peptide. Reactions are carried in the absence of metals and other reagents facilitating the purification and assuring a more bio environment. When multiple Lys are presented in peptide 3, the incorporation of the peptide can be carried out with the ε-amino functions of the additional Lys protected with an orthogonal protecting group,[21] which after conjugation can be removed. We envisage that this strategy will find broad applications for multidrug presentation and peptide-vaccine constructs.

Corresponding authors
Dr Beatriz G. de la Torre (beatrizgdelatorre@gmail.com) and Prof. Fernando Albericio (Falbericio@ukzn.ac.za, albericio@ub.edu)

Acknowledgments
This work was funded in part by the following: the National Research Foundation, the University of KwaZulu-Natal; Asphen Pharmacare (South Africa) and the CICYT (CTQ2012-30930); the Generalitat de Catalunya (2014 SGR 137). SR greatly acknowledges NRF-DST, SA for the award of Innovation Scholarship.

Notes and references

[a]*Catalysis and Peptide Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban 4001, South Africa*
[b]*School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4001, South Africa*
[c]*CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, 08028 Barcelona, Spain*
[d]*Department of Organic Chemistry, University of Barcelona, 08028-Barcelona, Spain*

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

1 X. Chen, B. Ren, M. Chen, M-X. Liu, W. Ren, Q-X. Wang, L-X. Zhang, G-Y. Yan, *PLoS One*, 2014, **9**, e86499.
2 K. Naghmouchi, C. L. Lay, J. Baah, D. Drider, *Res. Microbiol.,* 2012, **163**, 101.
3 H.F. Gaertner, F. Cerini, A. Kamath, A-F. Rochat, C-F. Siegrist, L. Menin, O. Hartley. *Bioconjugate Chem*., 2011, **22**, 1103.
4 M. Skwarczynski, I. Toth. *Nanomedicine* 2014, **9**, 2657.
5 S. Ramesh, P. Cherkupally, T. Govender, H. G. Kruger, F. Albericio and B. G. de la Torre, "Chemical platforms for peptide vaccine constructs", In: Advances in Protein Chemistry and Structural Biology, Ed: Rossen Donev, Elsevier, 2015, **99**, 99.
6 M. Goodman, W. Cai, Weibo, G.A. Kinberger. *Macromolecular Symposia* 2003, **201**, 223.
7 J. Hauert, J. Fernandez-Carneado, O. Michielin, S. Mathieu, D. Grell, M. Schapira, O. Spertini, M. Mutter, G. Tuchscherer, T. Kovacsovics. *ChemBioChem.,* 2004, **5**, 856.
8 M. Galibert, O. Renaudet, P. Dumy, D. Boturyn, *Angew. Chem., Int. Ed.,* 2011, 123, 1941.

9 A. P. Frei, O.-Y. Jeon, S. Kilcher, H. Moest, L. M. Henning, C. Jost, A. Pluckthun, J. Mercer, R. Aebersold, E. M. Carreira, B. Wollscheid, *Nat. Biotechnol*., 2012, 30, 997.

10 G. Viault, S. Dautrey, N. Maindron, J. Hardouin, P-Y. Renard, A. Romieu. *Org. Biomol. Chem.* 2013, 11, 2693

11 J. P. Tam, *Proc. Natl. Acad. Sci. U. S. A.*, 1988, **85**, 5409.

12 S. Ramesh, P. Cherkupally, T. Govender, H. G. Kruger, F. Albericio and B. G. de la Torre, *Org. Lett.*, 2015, **17**, 464.

13 P. Moody, M. E. B. Smith, C. P. Ryan, V. Chudasama, J. R. Baker, J. Molloy and S. Caddick, *ChemBioChem.*, 2012, **13**, 39.

14 M. W. Jones, R. A. Strickland, F. F. Schumacher, S. Caddick, J. R. Baker, M. I. Gibson and D. M. Haddleton, *Chem. Commun.*, 2012, **48**, 4064.

15 M. E. B. Smith, F. F. Schumacher, C. P. Ryan, L. M. Tedaldi, D. Papaioannou, G. Waksman, S. Caddick and J. R. Baker, *J. Am. Chem. Soc.*, 2010, **132**, 1960.

16 W. Kowalczyk, M. Monsó, B. G. de la Torre, D. Andreu. *J Pept Sci.,* 2011, **17**, 247.

17 M. Monsó, W. Kowalczyk, D. Andreu, B. G. de la Torre, *Org. Biomol. Chem*., 2012, **10**, 3116.

18 R. Subiros-Funosas, A. El-Faham and F. Albericio, *Org. Biomol. Chem.*, 2010, **8**, 3665.

19 R. Subiros-Funosas, R. Prohens, R. Barbas, A. El-Faham and F. Albericio, *Chem. - Eur. J.*, 2009, **15**, 9394.

20 F. Garcia-Martin, M. Quintanar-Audelo, Y. Garcia-Ramos, L. J. Cruz, C. Gravel, R. Furic, S. Cote, J. Tulla-Puche and F. Albericio, *J. Comb. Chem.*, 2006, **8**, 213.

21 A. Isidro-Llober, M. Alvarez, F. Albericio. *Chem. Rev.* 2009, **111,** 2455.